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Chlamydia trachomatis Inclusion Membrane Protein CT228 Recruits Elements of the Myosin Phosphatase Pathway to Regulate Release Mechanisms

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SUMMARY

Chlamydia trachomatis replicates within a membrane-bound compartment termed an inclusion. The inclusion membrane is modified by the insertion of multiple proteins known as Incs. In a yeast two-hybrid screen, an interaction was found between the inclusion membrane protein CT228 and MYPT1, a subunit of myosin phosphatase. MYPT1 was recruited peripherally around the inclusion, whereas the phosphorylated, inactive form was localized to active Src-family kinase-rich microdomains. Phosphorylated myosin light chain 2 (MLC2), myosin light chain kinase (MLCK), myosin IIA, and myosin IIB also colocalized with inactive MYPT1. The role of these proteins was examined in the context of host-cell exit mechanisms (i.e., cell lysis and extrusion of intact inclusions). Inhibition of myosin II or small interfering RNA depletion of myosin IIA, myosin IIB, MLC2, or MLCK reduced chlamydial extrusion, thus favoring lytic events as the primary means of release. These studies provide insights into the regulation of egress mechanisms by *C. trachomatis.*

INTRODUCTION

Chlamydiae are Gram-negative obligate intracellular bacteria that cause a variety of infections in humans and animals. *Chlamydia trachomatis* is comprised of multiple serological variants, or serovars, that are responsible for different diseases. Among these are the causative agents of the most prevalent bacterial sexually transmitted disease in the United States (Schachter, 1999) and the etiological agents of trachoma, the leading cause of infectious blindness worldwide (Burton and Mabey, 2009). All chlamydiae share common infection strategies, including a biphasic developmental cycle that consists of metabolically inactive infectious elementary bodies (EBs) and metabolically active and noninfectious reticulate bodies (RBs) (Moulder, 1991). The chlamydial developmental cycle occurs within a unique parasitophorous vacuole termed an inclusion (Hackstadt et al., 1997). At the end of the developmental cycle, EBs are released to initiate new cycles of infection. Release may be accomplished by lysis of the host cell or extrusion of intact inclusions by an active process requiring actin polymerization (Chin et al., 2012; Hybiske and Stephens, 2007; Todd and Caldwell, 1985).

The inclusion membrane is the interface for interaction between chlamydiae and the host cell. Early in infection, the inclusion membrane is extensively modified by the insertion of bacterially synthesized, type III secreted proteins, termed inclusion membrane proteins (Incs), that decorate the cytosolic face of the inclusion (Hackstadt et al., 1999; Rockey et al., 1997; Scidmore and Hackstadt, 2001). The number of Incs predicted varies among species, with predictions for *C. trachomatis* ranging between 36 and 59 Incs (Bannantine et al., 2000; Dehoux et al., 2011; Li et al., 2008; Lutter et al., 2012; Shaw et al., 2000; Toh et al., 2003). These Incs are comprised of a highly diverse set of proteins that are suspected of being involved in modulating host signaling pathways and cellular functions. Virtually all interactions of the chlamydial inclusion with the eukaryotic host cell require de novo chlamydial protein synthesis and, presumably, modification of the inclusion membrane (Scidmore et al., 1996). These functions include acquisition of host lipids (Carabeo et al., 2003; Hackstadt et al., 1996) and trafficking to the microtubule organizing center (MTOC), where the inclusion is typically observed in close proximity to the host cell nucleus and centrosomes (Clausen et al., 1997; Grieshaber et al., 2003, 2006; Higashi, 1965; Mital and Hackstadt, 2011; Mital et al., 2010). There are relatively few confirmed interactions of host proteins with specific Incs. These include the interactions of CT229 with Rab4 (Rzomp et al., 2006), IncG (CT118) recruitment of 14-3-3β (Scidmore and Hackstadt, 2001), and IncD (CT115) interaction with CERT (Derré et al., 2011).

In this study, a yeast two-hybrid screen of *C. trachomatis* Inc CT228 identified an interaction with myosin phosphatase target subunit 1 (MYPT1), a subunit of myosin phosphatase. MYPT1 was recruited peripherally around the chlamydial inclusion, whereas the phosphorylated, inactive form of MYPT1 was enriched at Src-family kinase-rich microdomains (Mital et al., 2010) on the inclusion membrane. Phosphorylated myosin light chain 2 (MLC2), myosin light chain kinase (MLCK), and myosin IIA and IIB were also identified in the inclusion microdomains.

Depletion of myosin IIA, myosin IIB, MLCK, or MLC2 by small interfering RNA (siRNA) resulted in decreased extrusion formation by *C. trachomatis*, suggesting that CT228 functions in regulation of release mechanisms at the end of the developmental cycle.

RESULTS

Yeast Two-Hybrid Screen of CT228

The C terminus of CT228 downstream of the bilobed hydrophobic domain (amino acids [aa] 86–196; Figure 1A) was cloned into Gal4 DNA binding domain (BD Bioscience) pGBKT7 (Clontech; Figure 1B) and transformed into yeast strain AH109. To identify putative interacting partners, AH109 containing pGBKT7-CT228 was used as bait to screen yeast strain Y187 containing a random normalized HeLa complementary DNA (cDNA) library (Clontech). A low-stringency screen was first employed to select for diploids and the results were confirmed in higher-stringency conditions on SD-Trp Leu His Ade + X-α-galactosidase plates. Prey plasmids were isolated and sequenced. One of the positive diploids identified after the bait dependency tests, Prey-43, contained the C terminus of the *PPP1R12A* gene, which corresponds to the leucine zipper domain of MYPT1. The interaction between CT228 and MYPT1 was verified in a targeted screen using in-frame fusions of Gal4AD to full-length MYPT1 (Figure 1C).

Coimmunoprecipitation experiments confirmed the interaction between CT228 and MYPT1 in vivo. MYPT1 was coprecipitated by anti-CT228 in the *C. trachomatis* L2-infected lysates, but not in uninfected lysates or by an irrelevant antiserum (Figure 1D).

To verify the domain of MYPT1 (Figure 1E) interacting with the C terminus of CT228, full-length and N-terminal truncations of MYPT1 starting at aa 445, 632, and 845 either with (MYPT1) or without the leucine zipper (MYPT1ΔLZ) were fused to Gal4AD and screened for interaction with CT228 (Figure 1F). The interaction of MYPT1 with the C terminus of CT228 was specific, as MYPT1 did not interact with the empty vector, pGADT7. The interaction of CT228 with MYPT1 was limited to the constructs that contained the leucine zipper, suggesting that the interaction was specific to that region.

CT228 and MYPT1 Localization to the Inclusion Membrane

HeLa cell monolayers infected with *C. trachomatis* L2 for 18 hr were stained with rabbit anti-CT228 and mouse anti-MOMP or mouse anti-Src pY419. CT228 was observed around the circumference of the inclusion membrane but was enriched in discrete microdomains colocalizing with active Src-family kinases (Figure 2A).

The distribution of MYPT1 in infected cells was also examined in *C. trachomatis* L2-infected HeLa cells. At 18 hr postinfection, cells were fixed and labeled with polyclonal rabbit anti-MYPT1 and mouse anti-MOMP (Figure 2B). MYPT1 was localized to the inclusion membrane and not the intracellular bacteria, similar to patterns previously observed for IncA and IncG (Hackstadt et al., 1999; Scidmore-Carlson et al., 1999). Dual labeling with MYPT1 and 14-3-3β, a host cell protein that was previously shown to be recruited to the inclusion membrane through its interaction with IncG (Scidmore and Hackstadt, 2001), demonstrated localization of MYPT1 to the inclusion membrane (Figure 2B). The recruitment patterns of MYPT1 to both the periphery of the inclusion and microdomains matches the localization of CT228. To confirm the recruitment of MYPT1 to the inclusion membrane, an N-terminal mCherry fusion of full-length MYPT1 was expressed in *C. trachomatis* L2-infected HeLa cells. In HeLa cells expressing mCherry-MYPT1, MYPT1 was recruited to the periphery of the inclusion membrane in the same pattern seen with MYPT1 antibodies (Figure 2C). The recruitment of MYPT1 to the periphery of the inclusion membrane with enrichment at microdomains was corroborated by immunoelectron microscopy using anti-MYPT1 with immunoperoxidase labeling to show the diaminobenzidine reaction product at the cytosolic face of the inclusion membrane (Figure 2D).

MYPT1 Recruitment Is Species Specific

The recruitment of MYPT1 to inclusions of other chlamydial serovars and chlamydiae species was examined. HeLa cells were infected with *C. muridarum*, *C. caviae*, *C. pneumoniae*, or the *C. trachomatis* serovars A, B, D, or L2, and stained with chlamydia lipopolysaccharide (LPS) and MYPT1 antibodies. MYPT1 localized to all *C. trachomatis* inclusions except serovar B/Jali20 (Figure 3), which encodes a truncated form of CT228 (Seth-Smith et al., 2009) and does not detectably express CT228. *C. muridarum* also recruited MYPT1 to the inclusion membrane, but *C. caviae* and *C. pneumoniae* did not, suggesting that there are diverse requirements for MYPT1 among the different chlamydial species and *C. trachomatis* serovars. The absence of MYPT1 from serovar B/Jali20, which does not express CT228, confirms the role of this inclusion membrane protein in recruitment of MYPT1.

C. trachomatis Infection Induces Phosphorylation Changes in MYPT1 and Selective Recruitment of pT853-MYPT1 to Chlamydial Microdomains

CT228 is one of the Incs that are transcribed early in infection (Belland et al., 2003; Shaw et al., 2000). By 4 hr postinfection, *C. trachomatis* has initiated protein synthesis and modified the inclusion membrane. Recruitment of MYPT1 is observed by this time (Figure 4A) and is retained circumferentially around the inclusion membrane for the duration of the developmental cycle.

MYPT1 is known to be phosphorylated by a number of kinases, specifically Rho-associated kinase (ROCK) at threonines 853 and 696 (Feng et al., 1999; Kawano et al., 1999). Phosphorylation of these sites is inhibitory to MYPT1 function due to tertiary folding changes to MYPT1 that no longer allow for the binding of the myosin phosphatase complex to MLC2. Dephosphorylation of MLC2 at threonine 18 and serine 19 by myosin phosphatase is inhibitory to myosin motor activity, and thus phosphorylation of MYPT1 favors MLC2 activity. Because the phosphorylation state of MYPT1 is important for activity, the phosphorylation of MYPT1 was investigated during *C. trachomatis* infection. Specific antibodies to MYPT1 phosphorylated at T853 (pT853) or T696 (pT696), as well as anti-MYPT1, were used to probe immunoblots of *C. trachomatis*-infected HeLa cell lysates taken at intervals

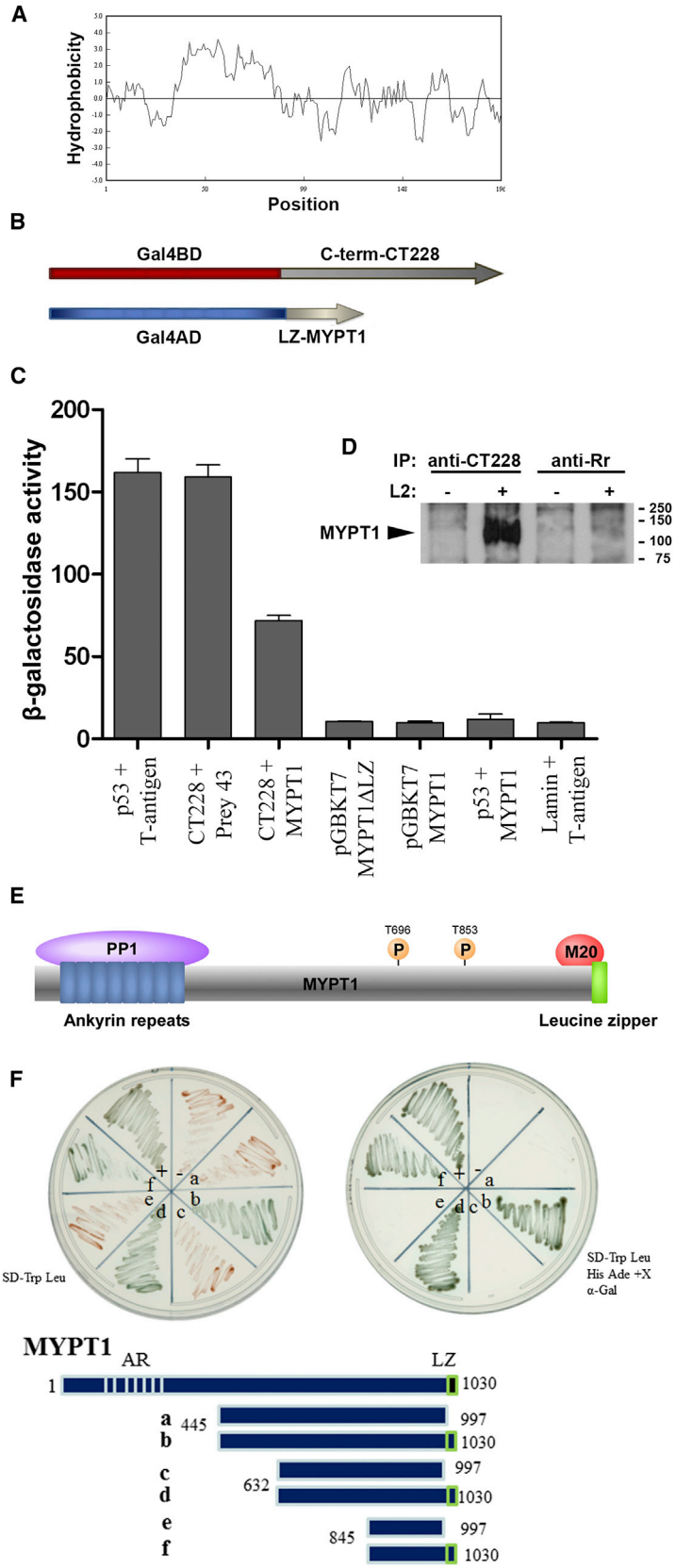

Figure 1. Yeast Two-Hybrid Screen and Interaction of *C. trachomatis* CT228 with MYPT1

(A) Kyte-Doolittle plot (Kyte and Doolittle, 1982) of CT228 showing the location of the bilobed hydrophobic domain.

(B) Schematic of bait and prey yeast-two hybrid constructs. The bait construct was generated by fusing the C terminus of CT228 in frame with Gal4BD. The interacting prey identified in the screen consisted of the leucine zipper of MYPT1 fused to Gal4AD (prey 43).

(C) Interactions in the yeast two-hybrid screen were verified by β-galactosidase assays of *S. cerevisiae* diploids generated from mating pairs of pGBKT7 (bait) and pGADT7 (prey) fusions. Liquid β-galactosidase assays confirmed interactions between the positive control p53 and T-antigen, CT228 (C terminus) and prey 43 (yeast two-hybrid prey containing the leucine zipper of MYPT1), and CT228 (C terminus) and full-length MYPT1. No interactions were detected with CT228 and MYPT1ΔLZ, pGBKT7 and MYPT1, or pGBKT7 and MYPT1ΔLZ, or in the negative control of Lamin and T-antigen. β-galactosidase activity was determined in triplicate, with error bars representing SD.

(D) Immunoblot of MYPT1 coimmunoprecipitated from L2-infected HeLa lysates with rabbit polyclonal antisera to CT228. No interaction was detected in uninfected HeLa lysates or the negative control antisera to *Rickettsia rickettsii* (anti-Rr).

(E) Diagram of myosin phosphatase showing the relevant regulatory phosphorylation sites on MYPT1, the small subunit (M20), and the catalytic subunit Protein Phosphatase 1 (PP1). Also shown are the locations of the ankyrin repeats and the leucine zipper.

(F) Interactions in the yeast two-hybrid screen were verified by α-galactosidase assays of *S. cerevisiae* diploids generated from mating pairs of GBKT7-CT228 and N-terminally truncated MYPT1 Gal4AD fusions (starting at aa 445, 632, or 845; either lacking or retaining the leucine zipper [a–f]). Diploids were grown in SD-Trp Leu and interactions were detected on high-stringency SD-Trp Leu His Ade + α-galactosidase. Interactions were detected between CT228 and MYPT1-Gal4AD fusions containing the leucine zipper (b, d, and f) as well as the positive control p53 + T-antigen (+). No interactions were detected with MYPT1-Gal4AD fusions lacking the leucine zipper (a, c, and e) and the negative control Lamin + T antigen (−). AR, ankyrin repeat; LZ, leucine zipper.

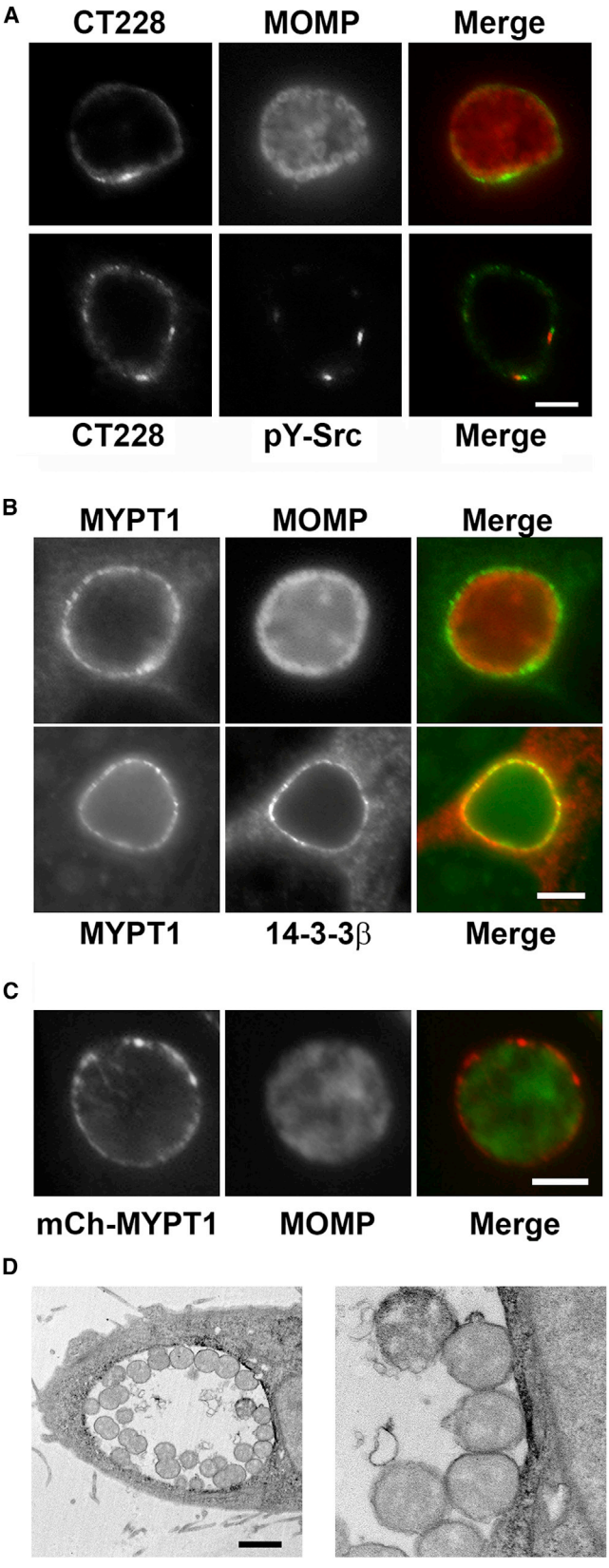

Figure 2. CT228 and MYPT1 Localization to the *C. trachomatis* Inclusion Membrane

(A) HeLa cell monolayers were infected with *C. trachomatis* L2 at an MOI of ~1 for 18 hr. Cells were fixed and labeled with anti-CT228 and anti-MOMP or anti-Src-pY419. Merged images show CT228 localizing to the periphery of the inclusion and enriched with active Src kinase. Scale bar, 5 μm.

(B) HeLa cells were infected at an MOI of ~1 for 18 hr, fixed, and labeled with rabbit anti-MYPT1 and mouse anti-*C. trachomatis* MOMP or mouse anti-14-3-3β.

(C) Expression of mCherry-MYPT1, showing recruitment to the *C. trachomatis* inclusion membrane at 18 hr postinfection. Scale bar, 5 μm (B and C).

(D) Immunoelectron microscopy showing localization of MYPT1 with enrichment at specific sites in the inclusion membrane. Scale bar, 2 μm.

throughout the developmental cycle (Figure 4B). Total MYPT1 levels remained constant in infected and uninfected lysates; however, by 42 hr postinfection, a dramatic reduction in phosphorylation of MYPT1 at both phosphorylation sites (T696 and T853) was seen in infected cell lysates.

To determine whether there was differential recruitment of the phosphorylated forms of MYPT1, *C. trachomatis* L2-infected HeLa cells were labeled with anti-pT853-MYPT1 and anti-Src-pY419, and examined by immunofluorescence. Phosphorylated MYPT1 was recruited to the inclusion, but was enriched in microdomains (Figure 4C) rather than circumferentially around the inclusion membrane as observed with the MYPT1 antibody (Figures 2 and 4A). The pattern was similar to that previously described for microdomains that are laden with active Src-family kinases (Mital et al., 2010). At 18 hr postinfection, pT853-MYPT1 was rarely observed at the microdomains. However, as the infection progressed, the pT853 form of MYPT1 became more prominent in the microdomains, suggesting a time-dependent phosphorylation change or recruitment of pT853-MYPT1 late in the chlamydial developmental cycle. Collectively, the data suggest that *C. trachomatis* infection results in decreased phosphorylation of the inhibitory sites on MYPT1, but selective recruitment of the remaining pT853-MYPT1 to microdomains on the chlamydial inclusion late in development. Thus, the phosphorylation and activity of MLC2 are expected to be enhanced at these focal points on the inclusion membrane at the time of egress.

Active MLCK, Serine 19 Phosphorylated MLC2, Myosin IIA, and Myosin IIB Are Recruited to Microdomains

When MYPT1 is phosphorylated at T853, its phosphatase activity is inhibited and correlates with increased levels of S19 phosphorylated MLC2. S19-phosphorylated MLC2 interacts with heavy-chain myosin II. Antagonistic to MYPT1 is MLCK, which actively phosphorylates MLC2. MLCK activity is also regulated by the phosphorylation state. Phosphorylation of Y464 (pY464) and Y471 (pY471) promotes kinase activity (Birukov et al., 2001). Because the inactive, phosphorylated T853 form of MYPT1 is recruited to inclusion membrane microdomains that are known to be areas of active Src-family kinase activity, the potential for MLCK, pS19-MLC2, and myosin II recruitment was also examined. *C. trachomatis* L2-infected HeLa cell monolayers were fixed and labeled with anti-Src-pY419 and either anti-pS19-MLC2, anti-pY464-MLCK, anti-pY471-MLCK, anti-myosin IIA, or myosin IIB (Figure 5). Each of these were enriched at inclusion microdomains and colocalized with active Src-family kinases.

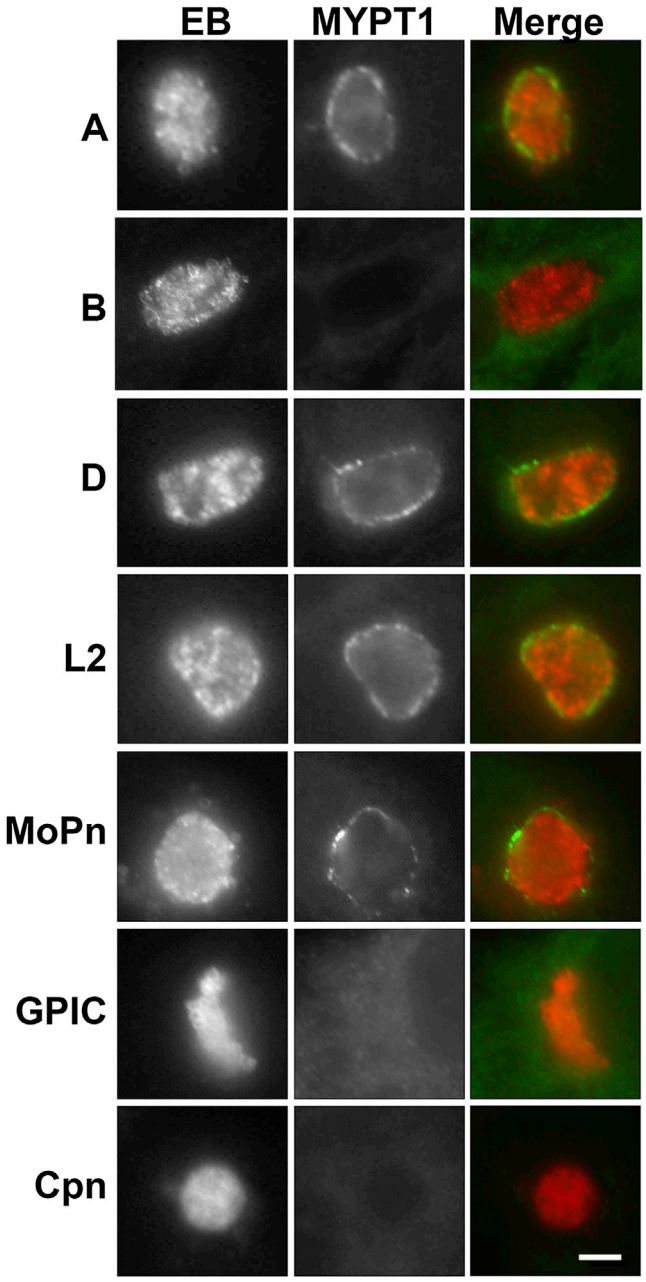

Figure 3. Species-Specific Recruitment of MYPT1

HeLa cells were infected with *C. trachomatis* serovars A, B, D, and L2; *C. muridarum* mouse pneumonitis (MoPn); *C. caviae* (GPIC); or *C. pneumoniae* AR-39 (Cpn). At specific times, cells were fixed (A: 30 hr; B: 30 hr; D: 30 hr; L2: 18 hr; MoPn: 30 hr; GPIC: 30 hr, and Cpn: 48 hr), permeabilized, and labeled with chlamydia LPS antibody and MYPT1 antibody. Chlamydial serovars and species grow at different rates. Images were selected to show inclusions of approximately the same size. MYPT1 recruitment was specific for *C. muridarum* and the *C. trachomatis* serovars A, D, and L2. Scale bar, 5 μm.

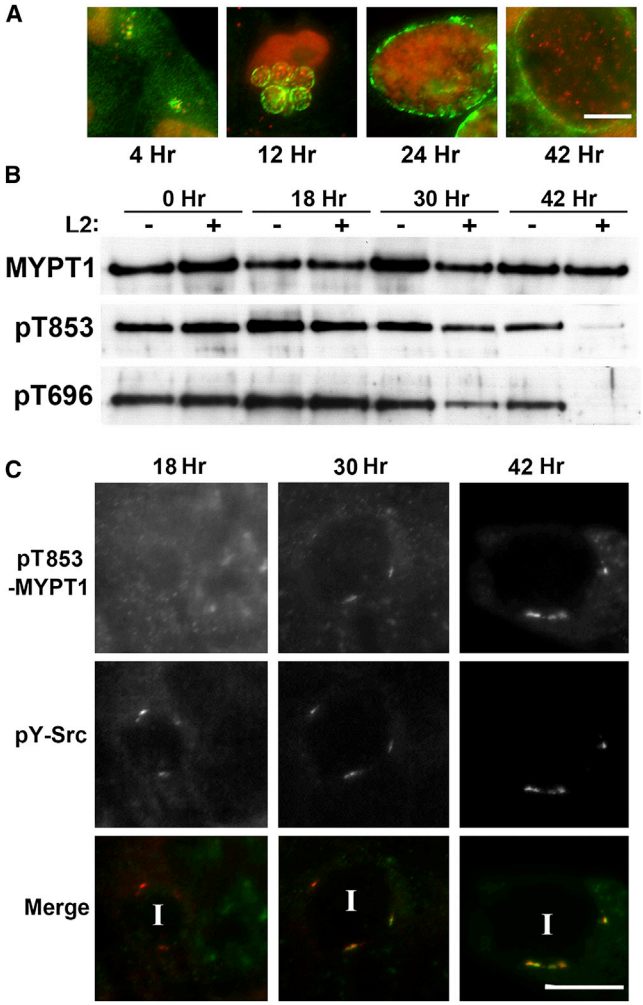

Figure 4. Infection by *C. trachomatis* L2 Causes Phosphorylation Changes in MYPT1 and Selective Time-Dependent Recruitment of pT853-MYPT1 in Microdomains

(A) Indirect immunofluorescence of *C. trachomatis* L2-infected HeLa cells at 4, 12, 24, and 42 hr postinfection at an MOI of ~10 stained with MYPT1 (green) and L2 EB (red) antisera to show early recruitment and retention of MYPT1 to the inclusion membrane. By 4 hr postinfection, the inclusion membrane has been modified by insertion of chlamydial protein, and trafficking of nascent inclusions to the MTOC is evident (Grieshaber et al., 2003). By 12 hr postinfection, replication of RBs is apparent but the inclusions have not yet fused into a single inclusion, as is typical of *C. trachomatis* (Hackstadt et al., 1999). Scale bar, 10 μm.

(B) Immunoblot analysis of cell lysates infected with *C. trachomatis* L2 at an MOI of ~5 for 0, 18, 30, and 42 hr showing levels of Pan-MYPT1, pT853-MYPT1, and pT696-MYPT1.

(C) Indirect immunofluorescence of *C. trachomatis* L2-infected HeLa cells at 18, 30, and 42 hr postinfection at an MOI of ~1 stained with anti-pT853-MYPT1 (green) and anti-Src-pY419 (red). The lumen of the inclusion (I) is indicated. Scale bar, 10 μm.

C. trachomatis Release by Extrusion Requires MLCK, MLC2, and Myosin II

Myosin II has been implicated in exit mechanisms of *C. trachomatis* at the end of the developmental cycle and is required for extrusion formation by *C. trachomatis* through the use of the inhibitor blebbistatin (Hybiske and Stephens, 2007); therefore, the role of the myosin phosphatase pathway in extrusion formation was investigated.

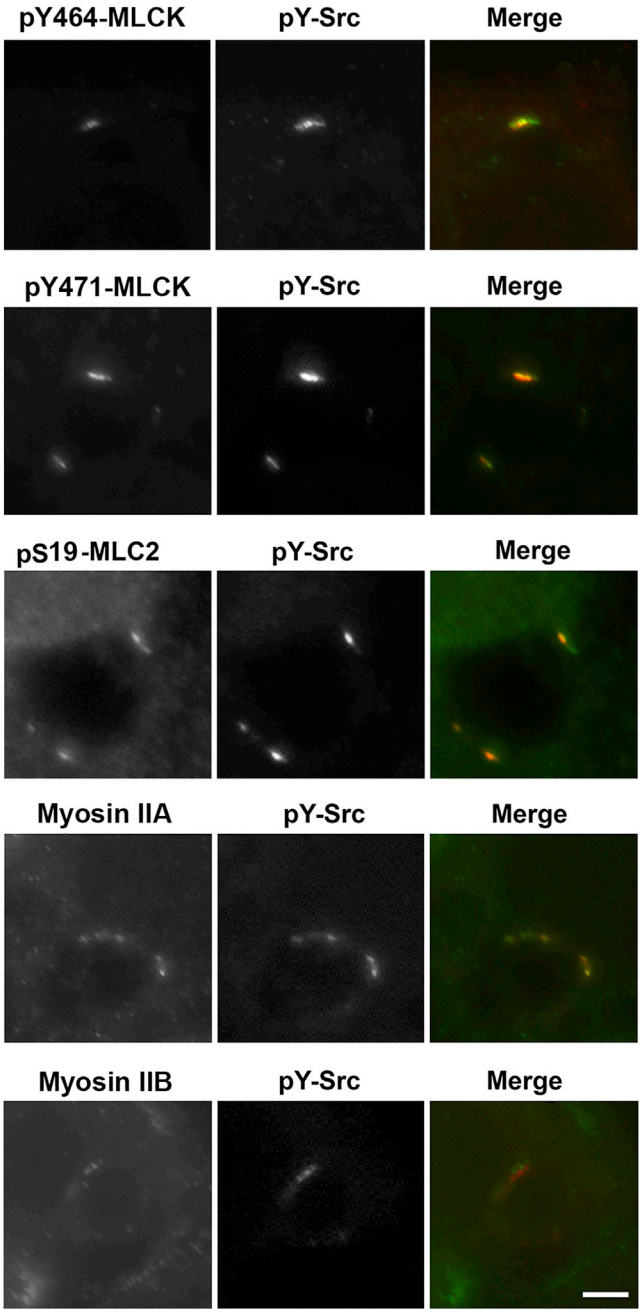

Figure 5. Active MLCK, S19-MLC2, Myosin IIA, and Myosin IIB Are Recruited to Microdomains on the Chlamydial Inclusion

Recruitment of pY464- and pY471-phosphorylated MLCK, S19-phosphorylated MLC2, and myosin IIA and IIB were examined by indirect immunofluorescence for colocalization with Src-pY419 in *C. trachomatis*-infected HeLa cells at 18 hr postinfection with an MOI of ~1. Representative images are shown. Scale bar, 5 μm.

Extrusion formation by *C. trachomatis* was monitored over time to identify peak times of extrusion formation and release. A peak of extrusion formation was seen at 48 hr postinfection (Figure S1).

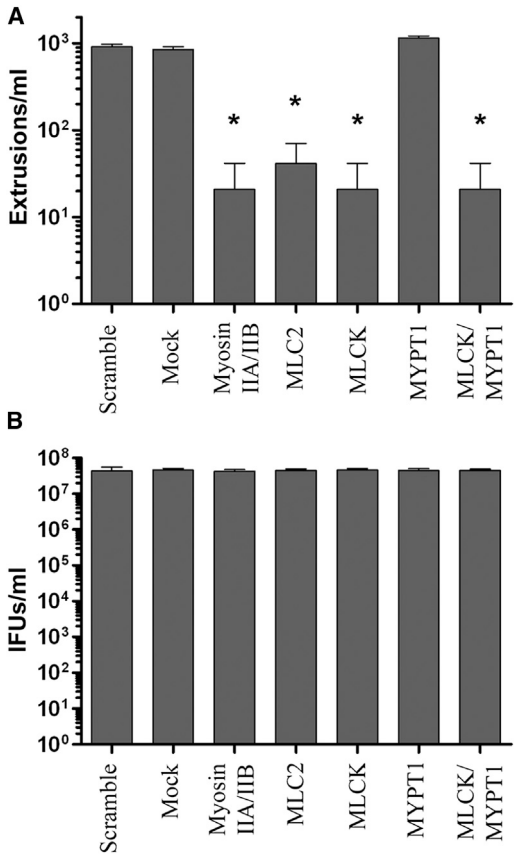

Figure 6. Depletion of MLCK, MLC2, Myosin IIA, and Myosin IIB Inhibits Extrusion Formation

(A) Effect on extrusion production by siRNA depletion of components of the myosin phosphatase and myosin kinase pathways.

(B) Effect on total infectious progeny production by siRNA depletion of components of the myosin phosphatase and myosin kinase pathways. Experiments were performed in triplicate; error bars represent SD. *$p < 0.0001$. See also Figure S1.

C. trachomatis-infected cells were also treated with DMSO, blebbistatin (myosin II inhibitor), or jasplakinolide (actin depolymerization inhibitor) and monitored for extrusion formation and total inclusion-forming units (IFUs) at 48 hr postinfection. As previously described (Hybiske and Stephens, 2007), blebbistatin significantly inhibited extrusion formation ($p < 0.0001$) compared with the jasplakinolide- or DMSO-treated cells (Figure S1). The reduced extrusion formation was not due to growth inhibition, since the total infectious plaque-forming units were constant under all conditions.

The effects on the release mechanisms of *C. trachomatis* by siRNA depletion of MLCK, MYPT1, MLC2, and myosin IIA and IIB were assessed. The efficacy of siRNA knockdowns was confirmed by QuantiGene analysis. All targeted messages were decreased by >75%. Depletion of MYPT1 had no effect on extrusion formation (Figure 6A). This result was not surprising because it is the inactive, phosphorylated form of MYPT1 that is associated with activation of the myosin motor complex, and thus absence of MYPT1 is indistinguishable from its inactivation.

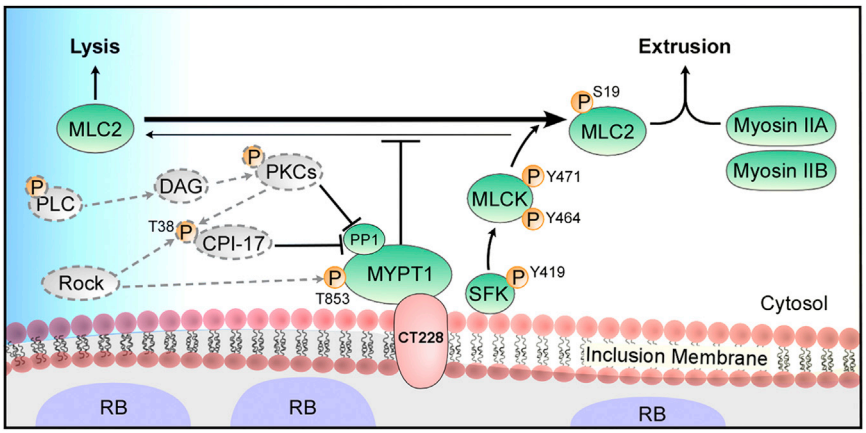

Figure 7. Schematic Representation of the Myosin Phosphatase Pathway in *C. trachomatis* Microdomains

The role of myosin phosphatase is to dephosphorylate MLC2. When phosphorylated at T853, MYPT1 is inhibited and can no longer dephosphorylate MLC2. Src-family kinases (SFK) are known to phosphorylate MLCK, which in turn actively phosphorylates MLC2. Myosin IIA, myosin IIB, and phosphorylated MLC2 and MLCK are required for *C. trachomatis* extrusion. Dashed lines are used to present potential inhibitory upstream regulators of MYPT1, including ROCK, PP1, C-kinase potentiated protein phosphatase-1 inhibitor (CPI-17), PKCs, diacylglycerol (DAG), and phospholipase C (PLC), that could contribute to myosin phosphatase inhibition and chlamydial extrusion.

However, depletion of MLCK, MLC2, and myosin IIA and IIB significantly reduced extrusion formation, suggesting that each of these proteins was essential for *C. trachomatis* to produce extrusions. Dual knockdowns of MYPT1 and MLCK produced levels of extrusion events similar to those observed with MLCK depletion alone. The extent of extrusion formation was not dependent on total progeny IFUs, as *C. trachomatis* produced similar levels of infectious progeny in all siRNA treatments (Figure 6B). The effects of MLCK, MYPT1, MLC2, MLCK and MYPT1 combined, and myosin IIA and IIB depletion on dynein-dependent interactions with the early inclusion were assessed by two independent assays that were previously used to analyze *C. trachomatis* inclusion trafficking (Mital and Hackstadt, 2011). None of the depletions altered early trafficking of nascent inclusions to the MTOC or the distance of mature inclusions from centrosomes (not shown). The interactions of the *C. trachomatis* inclusion with the myosin phosphatase pathway and the actin cytoskeleton do not appear to disrupt interactions with dynein and microtubule-dependent trafficking.

DISCUSSION

In a yeast two-hybrid screen for Inc-interacting host proteins, an interaction between the inclusion membrane protein CT228 and a subunit of myosin phosphatase, MYPT1, was observed. The recruitment of MYPT1 to the *C. trachomatis* inclusion was confirmed and showed a unique pattern of circumferential labeling of the inclusion membrane with enrichment at inclusion membrane microdomains. The activity of MYPT1 is regulated by the phosphorylation state (Amano et al., 1996). MYPT1 is phosphorylated by a number of kinases, including ROCK at T696 and T853 (Feng et al., 1999; Kawano et al., 1999), protein kinase C (PKC) at the ankyrin repeats (Tóth et al., 2000), integrin-linked kinase at T34 (Kiss et al., 2002), and Zip kinase at T695 (Haystead, 2005). The T696 and T853 sites are inhibitory because phosphorylation induces tertiary folding changes to MYPT1 that no longer allow for the binding of MLC2 and thus prevent the dephosphorylation of MLC2. MLC2 thus remains in an active state to interact with myosin IIA and myosin IIB to constitute an active myosin motor complex. The recruitment of T853-phosphorylated MYPT1 to chlamydial inclusion microdomains suggests that myosin phosphatase would be in an inactive state at these specific sites on the inclusion membrane. Accordingly, the myosin phosphatase substrate, MLC2, was observed in the S19-phosphorylated, active state within these Src-family kinase-rich microdomains. MLCK is the kinase that phosphorylates MLC2 at T18 and S19; however, only recruitment of pS19-MLC2 was addressed in our study. MLCK is activated by phosphorylation at Y464 and Y471 (Birukov et al., 2001), and recruitment of active MLCK to the chlamydial inclusion was confirmed. The enrichment of active Src-family kinases at *C. trachomatis* inclusion microdomains (Mital et al., 2010) suggests that they play an important role in the phosphorylation and activation of MLCK, thereby contributing to the phosphorylation of MLC2 and the activation of the myosin motor complex at inclusion membrane microdomains. Recently, myosin was implicated in one of the egress mechanisms of *C. trachomatis*, i.e., extrusion of intact inclusions from the host cell, rather than a lytic event (Hybiske and Stephens, 2007). In this work, we confirmed the recruitment of myosin IIA and myosin IIB to inclusion membrane microdomains. A model depicting key elements of the myosin regulatory pathway associated with the inclusion membrane is shown in Figure 7. In this model, the antagonistic activities of myosin phosphatase and myosin kinase would be expected to shift the balance of lytic versus extrusion egress mechanisms in response to external environmental stimuli and appropriate cellular signaling pathways.

All intracellular pathogens must eventually exit the host cell (Friedrich et al., 2012; Hybiske and Stephens, 2008). *C. trachomatis* has evolved at least two (and possibly three) distinct mechanisms of host-cell egress—cell lysis and extrusion (Hybiske and Stephens, 2007)—and a nonlytic, exocytosis-like mechanism (Todd and Caldwell, 1985). The extrusion mechanism is an actin-dependent process that involves protrusion of the inclusion from the host cell followed by constriction and resealing of the plasma membrane and subsequent release of the intact membrane-bound inclusion with survival of the host cell. The extrusion mechanism is believed to be dependent on actin polymerization, N-WASP, Rho GTPase, and myosin II through the use of specific inhibitors of each of these activities (Hybiske and Stephens, 2007). Recruitment of the actin coat to the inclusion prior to extrusion appears to be a sporadic and

dynamic event that relies on a combination of both bacterial and host factors (Chin et al., 2012).

C. trachomatis manipulates the myosin phosphatase pathway throughout the course of its developmental cycle. MYPT1 associates with CT228 at the inclusion membrane early and remains throughout the developmental cycle. In this state, MYPT1 is not phosphorylated and thus is an active MLC2 phosphatase that is expected to favor inactive myosin motor complexes at the inclusion membrane, which may prevent premature release of inclusions from the cell. This is in contrast to the localized enrichment of phosphorylated MYPT1 that occurs at discrete microdomains late in infection. A comparison of HeLa cell lysates from uninfected and infected cells shows an overall dephosphorylation of MYPT1 in the later stages of infection. Thus, the majority of the phosphorylated, inactive MYPT1 is localized at the inclusion membrane, where it appears to serve as focal point at which activation of MLC2 by MLCK is favored and the myosin motor complex is activated to promote egress of the intact chlamydial inclusion. The manipulation of MYPT1 phosphorylation by *C. trachomatis* not only limits active myosin complexes in the host cell but also permits selective exploitation of active myosin motor complexes by *Chlamydia* for egress.

Recruitment of MYPT1 among the different chlamydial species and serovars can be at least partially accounted for by the diversity of CT228. *C. trachomatis* serovar B/Jali20 encodes a truncated CT228 (Seth-Smith et al., 2009). Specifically, a deletion at position 86 of CT228 results in a frameshift that is predicted to produce a truncated CT228 (38 aa long) lacking the C terminus that interacts with the leucine zipper of MYPT1. We confirmed the lack of expression with specific antibodies. Accordingly, *C. trachomatis* B/Jali20 does not recruit MYPT1 to the inclusion membrane. Interestingly, *C. trachomatis* B/Jali20 did not form extrusions (not shown). The most closely related species to *C. trachomatis* that also recruited MYPT1, *C. muridarum*, contains a CT228 homolog that shares 50% identity at the amino acid level to CT228 of *C. trachomatis* L2. However, *C. muridarum* does not exhibit Src-kinase-rich microdomains (Mital et al., 2010). *C. caviae* and *C. pneumoniae* did not recruit MYPT1. *C. caviae* contains a distant homolog that may be too divergent to have retained the same function, and *C. pneumoniae* lacks a CT228 homolog (Lutter et al., 2012). It is likely that additional chlamydial or host factors, possibly those involved in the initiation of actin recruitment around the late inclusion (Hybiske and Stephens, 2007; Kumar and Valdivia, 2008), are required for the commencement of the actual extrusion process. Interestingly, neither MYPT1 nor inclusion membrane microdomains (Mital et al., 2010) are observed in *C. caviae*, although *C. caviae* has been described as utilizing the extrusion mechanism of release, albeit at an apparently reduced rate (Hybiske and Stephens, 2007). The recognition of *Chlamydia* variants lacking CT228 and/or Src-kinase-rich microdomains should be of value in deciphering the many potential regulatory pathways that control *C. trachomatis* egress from infected cells.

Modulation of MLC2 and MLCK phosphorylation states during the infection process of other pathogens has been described. MYPT1 had not been previously reported to be recruited to the site of infection of other intracellular pathogens, although changes in phosphorylation of MYPT1 were identified during *Trypanosoma cruzi* infection (Mott et al., 2009). Evidence of MLC2 phosphorylation during infection of enteropathogenic *E. coli* (EPEC) was observed in cell fractions associated with the cytoskeleton (Manjarrez-Hernandez et al., 1996). Alterations in intestinal epithelial cell permeability induced by either EPEC or enterohemorrhagic *E. coli* involve the phosphorylation of MLCK (Philpott et al., 1998; Yuhan et al., 1997). Myosin is a known target for other intracellular pathogens, including *Listeria monocytogenes*, which usurps myosin VIIA for uptake into host cells (Sousa et al., 2004), and *Shigella flexneri*, which requires myosin II (Rathman et al., 2000) for dissemination and myosin-X for filopodia formation (Bishai et al., 2013). The *Salmonella enterica* serovar Typhimurium also co-opts myosin II via SopB to regulate salmonella-containing vacuole dynamics such as positioning and stability (Wasylnka et al., 2008). Several viruses (Santangelo and Bao, 2007; van Leeuwen et al., 2002) also utilize myosin in egress from host cells, although these viruses are not located in a membrane-bound compartment.

C. trachomatis inclusion membrane microdomains were first identified as areas of focal recruitment of activated Src-family tyrosine kinases and the microtubular cytoskeleton (Mital et al., 2010). It now appears that microdomains are also focal points for interactions with the actin cytoskeleton and involvement in egress at the end of the developmental cycle. Inclusion membrane microdomains thus appear to be platforms for interactions with both the actin and microtubular cytoskeletons. Whether the two pathways interact cooperatively or antagonistically remains to be determined. The fact that Src-family tyrosine kinases and early expressed Incs have been implicated in the regulation of both early trafficking of the inclusion and egress suggests that antagonistic activities may hold egress mechanisms in check until an appropriate signal is received at the end of the developmental cycle. Tyrosine phosphorylation seems to play multiple, species-specific roles in early versus late interactions with the cytoskeleton throughout the chlamydial developmental cycle (Mital and Hackstadt, 2011) and may be a key regulator of egress mechanisms.

It is interesting that chlamydiae would have two independent means of egress. It is suspected that the underlying purpose of chlamydial extrusion may lie in evasion of innate or acquired immune responses (Hybiske and Stephens, 2007). The release of infectious EBs encapsulated within an intact inclusion membrane may allow the chlamydiae to evade local inflammatory mediators for subsequent release and infection of susceptible cells at more distal sites. Interestingly, two potential upstream regulators of the myosin phosphatase pathway, PKC and ROCK, are activated by pathways involved in immune responses and inflammation. Activation of PKC and extracellular signal-related kinases (ERKs) during EPEC infection of intestinal epithelial cells leads to activation of NF-κB and the proinflammatory response (Savkovic et al., 2003). In addition, endothelial cell permeability is increased by LPS and tumor necrosis factor alpha (TNF-α), which activate p38 and ERK1/2 mitogen-activated protein kinases, increase NF-κB signaling and ROCK-mediated phosphorylation of MYPT1, and lead to the accumulation of phosphorylated MLC2 (Xing and Birukova, 2010). The upstream events leading to the phosphorylation of MYPT1 and MLC2 at the chlamydial inclusion microdomains remain to be verified,

but activation of upstream regulators of the myosin phosphatase pathway during an immune or inflammatory response to chlamydial infection would be expected to shift the mechanism of host-cell exit to favor extrusion. The dissemination of membrane-bound infectious EBs to distant sites to avoid exposure to the harsh environment of local inflammatory responses would thus be favored. Future characterization of the pathway and regulatory components should provide insights leading to a greater understanding of how *C. trachomatis* forms extrusions and the role it plays in chlamydial pathogenesis.

EXPERIMENTAL PROCEDURES

Strains and Cell Culture

The *C. trachomatis* serovars L2 (LGV 434), D (UW3-Cx), B (Jali20/OT), and A (HAR-13); *C. muridarum* (MoPn); *C. pneumoniae* (AR-39); and *C. caviae* (GPIC) were propagated in HeLa 229 cells and purified by Renografin density gradient centrifugation as previously described (Caldwell et al., 1981). HeLa 229 cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS). Yeast strain Y187 was purchased from Clontech, and AH109 was provided by M.A. Scidmore.

Antibodies

Monoclonal antibody L2-I-45 against *C. trachomatis* L2 major outer membrane protein (MOMP) was kindly provided by H.D. Caldwell. GAL4-DNA BD fusions of CT228 were detected with Myc antibody (Cell Signaling). Anti-MYPT1 (United States Biological) and anti-T853 MYPT1 (Abnova) were used to detect pan-MYPT1 and Thr-853-phosphorylated MYPT1 by indirect immunofluorescence. Anti-MYPT1, anti-pT853 MYPT1, anti-pT696 MYPT1, anti-Myc, and anti-glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling) were used for immunoblotting. Anti-phospho (pSer-19)-MLC2 was purchased from Abcam. Anti-Src-pTyr419 (clone 9A6; Millipore) was used to detect active Src-family kinases. Anti-14-3-3β (clone H8), anti-MLCK (pTyr471), and anti-MLCK (pTyr464) were purchased from Santa Cruz Biotechnology. Anti-*Chlamydia* LPS (Thermo Scientific) was used to detect all species of chlamydiae, and a rabbit polyclonal anti-EB serum was used to detect *C. trachomatis*. Anti-myosin IIA and anti-myosin IIB were purchased from Thermo Scientific. Anti-mouse or anti-rabbit DyLight 594 and DyLight 488 (Jackson ImmunoResearch Laboratories) were used as secondaries for indirect immunofluorescence, and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG) and anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used for immunoblotting and immunoelectron microscopy as previously described (Scidmore-Carlson et al., 1999).

CT228 Antibody Production

Overnight cultures of BL21 (DE3) were subcultured at 1:200 in 600 ml of Luria-Bertani broth and grown to mid-exponential phase at 37°C. CT228-His expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM, with a further 4 hr of growth at 37°C. The culture was harvested by centrifugation at 5,000 rpm for 10 min and protein purification was carried out using the ProBond Purification System (Life Technologies) according to the manufacturer's instructions. Purified protein was quantitated using the Bradford protein assay (Bio-Rad) and dialyzed overnight against 100 mM NaCl. The purified protein was emulsified in Sigma Adjuvant System (Sigma) and rabbits were immunized according to the manufacturer's instructions.

Yeast Two-Hybrid Library Screening

Yeast two-hybrid assays were performed with the use of the Matchmaker Gold yeast two-hybrid system (Clontech). The Y187 yeast strain, pretransformed with Normalized Mate and Plate HeLa S3 Library constructed in pGADT7-RecAB, was purchased from Clontech. pGBKT7-CT228 was transformed into AH109 and mated overnight with Y187 containing Normalized Mate and Plate HeLa S3 Library. Matings were screened for interacting clones by selecting diploids on SD plates lacking leucine (Leu), tryptophan (Trp), and histidine (His) (low stringency). Positive diploids were rescreened for the ability to grow on SD plates lacking His, Leu, Trp, and adenine (Ade) + X-α galactosidase (high stringency). Plasmid DNA was isolated from blue diploid colonies using the PrepEase Yeast Plasmid Isolation Kit (Affymetrix), transformed into *E. coli* XL1-blue (Stratagene), re-extracted using a QIAGEN plasmid extraction kit, and sequenced at RML Genomics, Research Technologies Section, National Institute of Allergy and Infectious Diseases. Bait dependencies and targeted screens were performed by directed mating between AH109 carrying pGBKT7-CT228 and Y187 carrying prey plasmids from selected diploids. Matings were plated on high-stringency plates and monitored for diploid growth. See the Extended Experimental Procedures for a detailed description of plasmid construction.

Protein Preparation and Western Blotting

Protein extracts from AH109 carrying pGBKT7-CT228 were prepared according to the urea/SDS protein extraction method provided in the Yeast Protocols Handbook (Clontech) prior to SDS-PAGE and electrophoretic transfer to nitrocellulose. HeLa cells were infected with *C. trachomatis* at a multiplicity of infection (moi) of ~1. At appropriate times postinfection, the cells were rinsed in PBS (pH 7.4) and lysed in Laemmli buffer (Laemmli, 1970) with 5% β-mercaptoethanol plus PhosSTOP inhibitor cocktail (Roche) and Complete Mini protease inhibitor cocktail (Roche). Protein extracts and cell lysates were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dry milk/2% BSA in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20 (TBS-T) and incubated with protein-specific primary antibody in blocking buffer overnight. Unbound antibody was removed by three rinses in TBS-T, and bound antibody was detected with HRP-conjugated donkey anti-rabbit IgG secondary. Blots were then rinsed three times in TBS-T and developed with Amersham ECL selection western blotting detection reagent (GE Healthcare Life Sciences) and exposed to CL-XPosure film (Thermo Scientific).

siRNA Knockdown

Monolayers of HeLa 229 cells (50% confluence) were plated on glass coverslips or Costar CellBIND (Corning) 24-well plates and incubated overnight with MYPT1, MLC2, myosin IIA, myosin IIB, MLCK, or Scramble Targetplus Smartpool siRNA (Dharmacon) complexed in DharmaFECT1 in RPMI with 10% FBS. At 72 hr posttransfection, cells were infected with *C. trachomatis* L2 at an MOI of 1 for 48 hr. Knockdown efficiency based upon transcript level was determined by QuantiGene analysis (Panomics) per the manufacturer's instructions. Lysates were probed with MYPT1, MLC2, myosin IIA, myosin IIB, MLCK, or actin-specific RNA probes, and the luminescence for each sample and probe was measured using a Synergy4 plate reader (BioTek Instruments). Each transcript was normalized to the actin signal as a loading control.

Coimmunoprecipitation

HeLa cells were either mock infected or infected with *C. trachomatis* L2. At 48 hr postinfection, the cells were washed three times with PBS, scraped into 0.75 ml of RIPA buffer plus PhosSTOP inhibitor cocktail (Roche) and Complete Mini protease inhibitor cocktail (Roche), and incubated for 20 min on ice. Cell debris was removed by centrifugation (13,000 rpm, 5 min, 4°C). Cell-free lysates were incubated with anti-CT228 or anti-Rickettsia for 1 hr with rotation at 4°C. Protein-A beads (Thermo Scientific) were added and rotation continued overnight at 4°C. The beads were washed three times with PBS and bound proteins were eluted into Laemmli buffer (Laemmli, 1970) with 5% β-mercaptoethanol, separated by SDS-PAGE, and electrophoretically transferred to nitrocellulose for detection with MYPT1 antibody.

Infectious Progeny and Inclusion Development Assays

Following siRNA knockdown and 48 hr of *C. trachomatis* L2 infection, both supernatant and cell lysates were examined for IFUs. Supernatant and cells lysed in distilled water were serially diluted in Hank's balanced salt solution and plated onto HeLa cell monolayers. After 24 hr of infection, monolayers were fixed in methanol and stained with rabbit anti-EB sera followed by anti-rabbit DyLight 488. The numbers of inclusions per field of view were counted

on 20 fields for each sample using a Nikon Eclipse 80i fluorescent microscope. Total IFUs/ml were calculated for each sample.

Extruded Inclusion Enumeration

Extruded inclusions were enumerated by a method similar to that described by Chin et al. (2012). HeLa cells were infected with *C. trachomatis* L2 at an MOI of ~1. At 24 hr postinfection, the medium was replaced with RPMI 1640 medium with 10% FBS supplemented with DMSO, blebbistatin (50 μM; EMD Millipore), or jasplakinolide (1 μM; Life Technologies). At the indicated times, the supernatants were removed and gently pelleted by centrifugation at 1,000 rpm. The resulting pellet was resuspended in 100 μl of media, mixed with trypan blue (Life Technologies), and stained with NucBlue Hoechst live-cell stain (Life Technologies). Extruded inclusions free of host cell nuclei were enumerated using a Hausser Bright-line Phase hemacytometer and a Zeiss LSM 510 Meta laser confocal scanning microscope.

Statistics

Statistical analysis was performed in Prism 5.0 using a one-way ANOVA and a Newman-Keuls posttest.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.027.

LICENSING INFORMATION

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